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Inhibition of Potentially Pathogenic Yeastlike Fungi by Clotrimazole in Combination with 5-Fluorocytosine or Amphotericin B

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Clotrimazole (CTM) has a doubtful future with respect to use in treatment of the systemic mycoses. To assess the potential of CTM in combined drug regimens, antifungal effects of CTM together with 5-fluorocytosine (5-FC) or amphotericin B (AMB) were tested in a synthetic liquid medium against *Candida albicans*, *Candida tropicalis*, and *Torulopsis glabrata*. Viable counts were monitored over a 48-h incubation period. Weak inhibitory concentrations of CTM were tested in combination with levels of 5-FC or AMB that alone produced transient antifungal effects followed by rapid recovery of proliferative capacity. Results were similar for each of the organisms studied. Between 24 and 48 h, when cultures containing 5-FC or AMB alone were in the recovery phase, CTM plus 5-FC and CTM plus AMB continued to markedly suppress cell multiplication. It would appear that weak inhibitory concentrations of CTM can act together with 5-FC or AMB to produce antifungal effects greater than that obtained with either of the latter two drugs alone.

Clotrimazole (CTM) is a relatively new synthetic antifungal drug (5, 6) that displays inhibitory activity against a broad spectrum of yeasts and filamentous fungi in vitro (3, 5–7, 11). However, results of studies designed to test the therapeutic effects of CTM in vivo have been inconsistent (5, 6, 9, 11). Since there is a lack of sufficient convincing data with respect to the clinical efficacy of CTM, and since severe gastrointestinal disturbances are often associated with its use (1, 2, 10, 12), CTM is not generally recommended for treatment of human systemic mycotic infections unless all other attempts to manage particular cases have failed (10, 12). Pharmacological studies have indicated that CTM is poorly and erratically absorbed from the human gastrointestinal tract (1, 3, 12). Based on a microbiological assay for CTM, Burgess and Bodey showed that after a 1.5- to 3.0-g oral dose peak serum concentrations were in the 1- to 2-μg/ml range (1), which borders on the minimal inhibitory concentration for many fungi (3, 6, 7, 11). As a further complication, it was observed that peak values progressively declined as therapy was continued for several days (1). These factors probably account for the disparity between in vitro and in vivo results with CTM.

In a preliminary study, Hoeprich and Finn (Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, Mm42, p. 135) reported that combinations of amphotericin B (AMB) and CTM were antagonistic when tested against isolates of *Candida albicans* and *Cryptococcus neoformans* in vitro. To our knowledge there is at present no other published information regarding the activity of this drug pair against yeastlike fungi of medical interest. This is surprising since there has been considerable recent interest in the use of combined drug therapy to treat systemic mycotic infections. The present study was designed to assess the potential of CTM in combined drug regimens. We have reexamined the in vitro activity of CTM-AMB combinations against three potentially pathogenic yeast species. In addition, we have preliminarily evaluated antifungal effects of CTM in combination with 5-fluorocytosine (5-FC) against the same test organisms.

MATERIALS AND METHODS

Organisms. *Candida albicans* (ATCC 11651) was purchased from the American Type Culture Collection, Rockville, Md. *Candida tropicalis* was given to us by J. Prince, Department of Microbiology, Univ. of Minnesota, Minneapolis. *Torulopsis glabrata* was provided by D. Serstock, Clinical Laboratory Service, Minneapolis Veterans Administration Hospital. The yeasts were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.).

Drugs. AMB was purchased from Calbiochem,

Los Angeles, Calif. CTM (Bay b 5097, batch 4820-42) was a gift from Delbay Pharmaceuticals, Inc., Bloomfield, N.J. Stock solutions of AMB and CTM were prepared in dimethyl sulfoxide ($MeSO_2$) several hours prior to each experiment. 5-FC was a gift from Hoffmann-La Roche, Inc., Nutley, N.J. Solutions of 5-FC were prepared in distilled water and sterilized by filtration.

Culture medium. The synthetic liquid medium used in these studies had the same composition as that described by Shadomy (8) and contained 6.7 g of yeast nitrogen base (Difco), 1.5 g of L-asparagine, and 10.0 g of dextrose in 1 liter of distilled water. For our purposes, however, the medium was adjusted to pH 7 before filter sterilization.

Experimental. Drugs were added to 20-ml volumes of synthetic liquid medium in 50-ml Erlenmeyer flasks. The total volume of drug solution or solutions added never exceeded 0.2 ml. $MeSO_2$ alone at a level of 0.5 ml per 20 ml of culture had no apparent effect on either the rate or extent of growth for any of the organisms studied. Inoculum cells were grown for 24 h at 37 C with rotary shaking at 150 rpm. Each experimental flask received a volume of inoculum culture sufficient to give a time zero cell density of 2×10^5 to 5×10^5 colony-forming units/ml. Actual time zero counts were verified by viability determinations. Experimental cultures were incubated at 37 C with rotary shaking in the dark. Standard dilution and plate count techniques were used to monitor viability over a 48-h period. Diluent blanks consisted of 0.9% saline containing 0.02% Tween 80. Pour plates were prepared with Sabouraud dextrose agar. All samples were plated in duplicate, incubated for 2 to 3 days at 30 C, and counted.

RESULTS

Concentrations of CTM used in these studies gave weak or partial fungistatic effects, as evidenced by downward shifts in logarithmic growth rates, and approximated maximal levels reported attainable in human serum (1). The concentrations of 5-FC and AMB selected were those that alone produced marked transient antifungal effects during the first 24 h, followed by rapid recovery of proliferative capacity between 24 and 48 h. Antifungal activities of the drugs both singly and in combination against *C. albicans* are shown in Fig. 1. During the first 24 h CTM plus 5-FC (Fig. 1A) and CTM plus AMB (Fig. 1B) were no more effective than either 5-FC or AMB alone. Between 24 and 48 h, however, both combinations continued to suppress multiplication, whereas those cultures containing 5-FC or AMB alone recovered. Recovery was evidenced by rapid proliferation resulting in 48-h cell densities that approximated maximal populations observed in the no-drug control cultures at 17 h. Similar results were obtained when the drugs were tested alone and in combination against *C. tropicalis* (Fig. 2). A concentration of only 0.10 μg of 5-FC per ml exerted significant fungicidal activity on *T. glabrata* during the early hours of drug exposure (Fig. 3A). Between 17 and 41 h, however, the survivors recovered and underwent rapid multiplication. A weak fungistatic concentration of CTM in combination with 5-FC had an even more pronounced fungicidal effect than did 5-FC alone. Although recovery of the survivors was quite rapid in the culture treated with both drugs, the number of colony-forming units per milliliter at 48 h was approximately two logs less than in the culture containing only 5-FC. Note in Fig. 3B that during the first 24 h AMB alone was moderately fungicidal

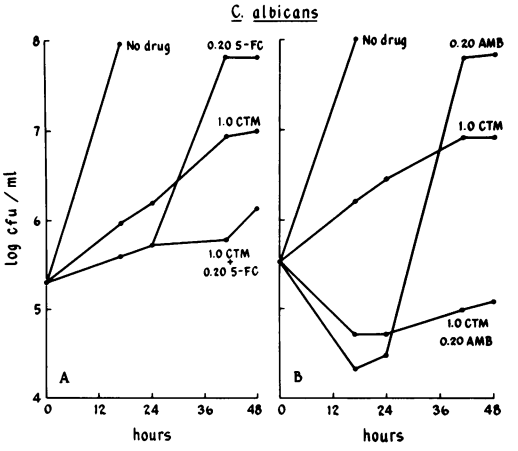

FIG. 1. *Antifungal activities of CTM plus 5-FC (A) and CTM plus AMB (B) in relation to single-drug effects against C. albicans. Numbers preceding drug abbreviations are micrograms of drug per milliliter.*

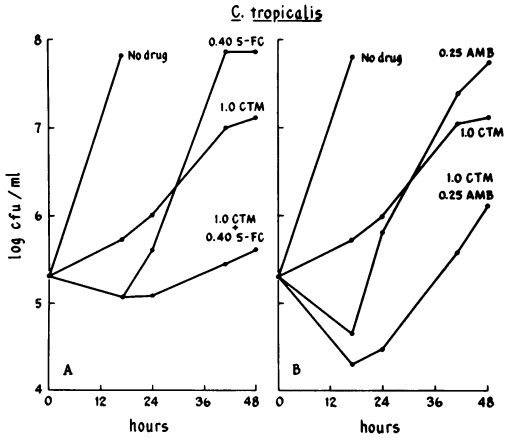

FIG. 2. *Antifungal activities of CTM plus 5-FC (A) and CTM plus AMB (B) in relation to single-drug effects against C. tropicalis. Numbers preceding drug abbreviations are micrograms of drug per milliliter.*

against *T. glabrata*, but the combination of CTM plus AMB was only fungistatic. However, between 24 and 48 h the combination continued to suppress multiplication, whereas survivors in the AMB culture quickly recovered proliferative capacity.

DISCUSSION

It is always tempting to try to classify effects of combined drug activity as synergistic, additive, indifferent, or antagonistic (4). There were no indications of drug antagonism in *C. albicans* and *C. tropicalis* experiments. With *T. glabrata*, CTM did antagonize the fungicidal activity of AMB during the first 24 h of incubation. Instead of a lethal effect, fungistasis was observed (Fig. 3B). Between 24 and 48 h, however, capacity for cell multiplication was quickly regained in the culture containing AMB only, but continued to be suppressed in the presence of both drugs. When CTM plus 5-FC was tested against *T. glabrata* (Fig. 3A), the fungicidal effect observed met several of the criteria for antibiotic synergism established by Jawetz (4). With these exceptions, the antifungal activities of CTM plus 5-FC and CTM plus AMB combinations did not fit neatly into specific categories.

The primary concern in this investigation was not with the classification of drug interactions but rather with a comparison of single drug versus combined drug effects on total cell populations during relatively long incubation periods. In our system, a 48-h incubation period can be considered as prolonged in view of the fact that maximal cell populations were reached in drug-free control cultures by 17 h. Based on the results of in vitro studies with three different species of potentially pathogenic yeasts, it would appear that weak or partially inhibitory concentrations of CTM can act together with either 5-FC or AMB to produce antifungal effects in excess of those obtained with either of the latter two drugs alone. In the presence of CTM plus 5-FC or CTM plus AMB the capacity for rapid cell multiplication continued to be suppressed during the period that cultures containing only one drug were quickly regaining this capability. Both combinations markedly delayed recovery, and in this sense CTM appeared to enhance the antifungal activities of both 5-FC and AMB.

The results of this investigation raise many questions and numerous possibilities in regard to the design of combined drug regimens. We feel that both combinations merit further study and evaluation.

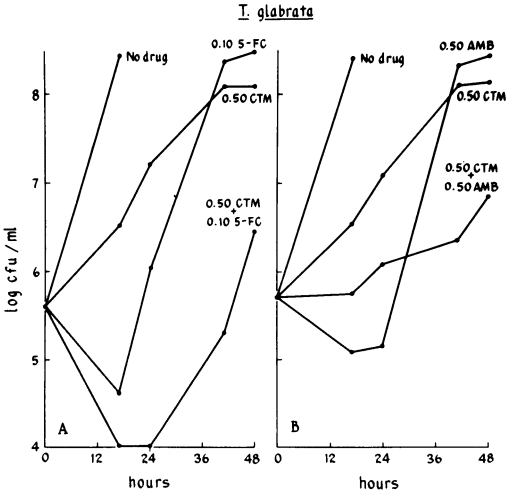

FIG. 3. *Antifungal activities of CTM plus 5-FC (A) and CTM plus AMB (B) in relation to single-drug effects against T. glabrata. Numbers preceding drug abbreviations are micrograms of drug per ml.*

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